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Article

Fate of Thymol and Its Monoglucosides in the Gastrointestinal Tract of Piglets

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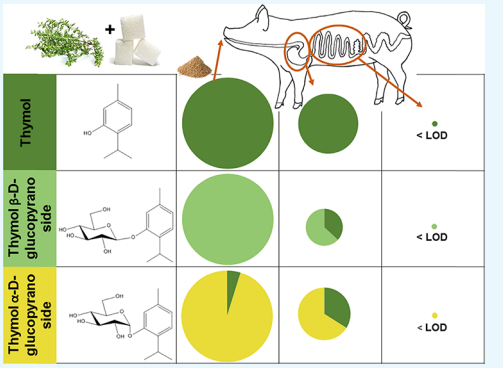
ABSTRACT: The monoterpene thymol has been proposed as a valuable alternative to in-feed antibiotics in animal production. However, the effectiveness of the antimicrobial is comprised by its fast absorption in the upper gastrointestinal tract. In this work, two glucoconjugates, thymol α-D-glucopyranoside (TαG) and thymol β-D-glucopyranoside (TβG), were compared with free thymol for their potential to deliver higher concentrations of the active compound to the distal small intestine of supplemented piglets. Additionally, an analytical method was developed and validated for the simultaneous quantification of thymol and its glucoconjugates in different matrices. In stomach contents of pigs fed with 3333 μmol kg^{-1} thymol, TαG, or TβG, total thymol concentrations amounted to 3048, 2357, and 1820 μmol kg^{-1} dry matter, respectively. In glucoconjugate-fed pigs, over 30% of this concentration was present in the unconjugated form, suggesting partial hydrolysis in the stomach. No quantifiable levels of thymol or glucoconjugates were detected in the small intestine or cecum for any treatment, indicating that conjugation with one glucose unit did not sufficiently protect thymol from early absorption.



INTRODUCTION

Thymol (2-isopropyl-5-methylphenol, Figure 1) is the main monoterpene component of the essential oil derived from *Thymus* species. The phenolic compound exhibits strong antimicrobial and antioxidant properties.[1] Therefore, it has been used in human medicine and the food industry for centuries.[2] More recently, phytochemicals received increasing interest for their application in animal nutrition due to the need for a reduction in the use of antibiotics. Thymol has been suggested as a valuable alternative for these in-feed antibiotics, especially for weaned piglets as weaning is a stressful event often associated with dysbiosis of gut microbiota and proliferation of pathogens, such as enterotoxigenic *Escherichia coli*, particularly in the distal small intestine.[3] Although the bactericidal capacity of thymol has been extensively demonstrated *in vitro*,[1,4−6] its application in animal feeds has several limitations. Next to its volatility and pungent taste at high concentrations, the main restriction is the fast and complete absorption of thymol in the upper gastrointestinal tract (GIT), resulting in insignificant concentrations at the level of the lower GIT, which are insufficient for the desired antibacterial effects.[7,8] Hence, protection of the molecule is opportune to obtain effective luminal concentrations in the distal section of the small intestine.

Encapsulation is a popular measure to increase the stability and functional performance of essential oils. A wide array of matrices (e.g., whey proteins, triglycerides, and maltodextrins) and techniques (e.g., spray drying and extrusion) are available, each with their advantages and drawbacks.[9,10] However, the existing encapsulations of essential oils are primarily designed for food and pharmaceutical applications.[9] Many of the encapsulated feed additives are still in the laboratory stage,[10] and only few formulations have been validated in pigs, with variable success in enhancing intestinal release.[11−13] Moreover, application of this approach for animal nutrition is limited by cost considerations.[14]

Glycosylation is an alternative protective method that has been used for various pharmaceuticals in the past, thus creating prodrugs.[15] It also reduces the volatility of the aglycon[16] and masks the pungent taste of thymol,[17] which is advantageous for application in animal feeds. Enzymatic glycosylation uses cheap resources (e.g., sucrose) and offers the possibility to synthesize an array of glycoconjugates with various sugar moieties in the form of mono-, di-, or polyglycosides[18] Moreover, a large variety of plant secondary metabolites naturally occur as

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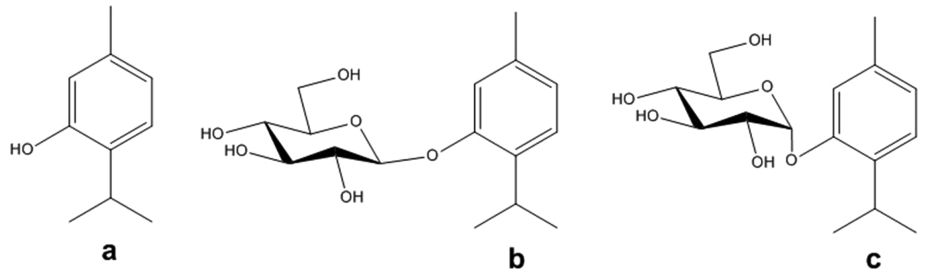

Figure 1. Chemical structures of (a) thymol, (b) thymol β-D-glucopyranoside, and (c) thymol α-D-glucopyranoside

Table 1. Parameters of Calibration Curves, Limit of Detection (LOD), and Limit of Quantification (LOQ) of Thymol and Thymol α-D-Glucopyranoside in Different Matrices

matrix	regression equation	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
thymol				
feed	$y = 0.0086x - 0.0538$	0.9962	3.5	10.6
gastric digesta	$y = 0.0106x - 0.0877$	0.9906	5.8	17.6
small intestinal digesta	$y = 0.0102x - 0.0007$	0.9943	6.4	19.5
cecal digesta	$y = 0.0094x - 0.0064$	0.9968	3.9	11.9
thymol α-D-glucopyranoside				
feed	$y = 0.0035x + 0.0203$	0.9982	5.7	17.3
gastric digesta	$y = 0.0045x + 0.0441$	0.9962	8.6	26.1
small intestinal digesta	$y = 0.0040x + 0.0058$	0.9991	5.5	16.6
cecal digesta	$y = 0.0040x + 0.0265$	0.9979	7.7	23.3

glycosides, increasing their water solubility and altering their biological activity.[19] The functionality of these glycoside prodrugs depends on the release of the active aglycon at the target site by cleavage of the glycosidic bond by glycoside-hydrolyzing enzymes. These glycosidases are present in the GIT of mammals and are either of endogenous or bacterial origin.[15]

To date, only one glycoside of thymol, namely, thymol β-D-glucopyranoside (TβG), has been investigated as a protected form of thymol in animal nutrition. Petrujkic et al.[20] compared the absorption of thymol and TβG in everted jejunal segments of the pig and concluded that the glucoconjugate was 2.3 and 2.8 times more resistant to absorption than pure thymol from 1 and 3 mmol L^{-1} solutions, respectively. Furthermore, oral administration of TβG resulted in significant reduction of the *Campylobacter* level in the crop of market-aged broilers, whereas pure thymol did not, as compared to a negative control.[21] However, TβG has not been evaluated *in vivo* as a feed additive in pigs.

This work focuses on thymol α-D-glucopyranoside (TαG), the anomer of TβG (Figure 1), which has, to the best of our knowledge, not yet been investigated for its potential to deliver thymol to the distal small intestine. It is hereby assumed that there is sufficient glycosidase activity present for gradual release of the active aglycon thymol along the GIT. First, an analytical method was developed and validated for the simultaneous quantification of thymol and TαG in different matrices. Thereafter, an *in vivo* feeding trial was conducted to test and compare the retention of TαG along the GIT of piglets with that of free thymol and its anomer TβG.

RESULTS

Method Validation for the Analysis of Thymol and TαG. *Selectivity.* The method is considered selective as no interfering peaks originating from the matrix could be identified at the retention times of the peaks of interest, being 10.2, 17.2, 22.0, and 23.9 min for pNPG, TαG, iPP, and thymol, respectively.

Linearity. The calibration curves, expressed as the ratio of the peak areas of the analyte and its respective internal standard versus concentration, were found to be linear for both thymol and TαG over the concentration range of 5−1200 mg kg^{-1} for all matrices tested. Regression equations and determination coefficients (R^2) are displayed in Table 1.

Accuracy, Precision, and Recovery. The values for the validation parameters accuracy, precision, and recovery are listed in Table 2. The method can be considered accurate and precise. The accuracy ranged from 84.7 to 107.6% and 87.6 to 108.2% for thymol and TαG, respectively, at three concentrations in four different matrices. Recoveries were in the range of 95 to 118% for thymol and 95 to 122% for TαG. The highest values were found in feed at a dose of 100 mg kg^{-1}, indicating possible overestimation of the analyte concentrations in feed. This should, however, not pose a problem in the current study as concentrations added to the feed of the *in vivo* trial were 5 to 10 times higher.

Sensitivity. The LOD and LOQ are key parameters in method validation as they determine the lowest concentration of the analyte, which can be reliably distinguished from the baseline or quantitated, respectively. The values for LOD and LOQ are presented in Table 1.

Based on the obtained validation parameters presented above, the method is considered satisfactory for its application on *in vivo* samples.

Concentrations of Thymol and Its Glucosides in the GIT *In Vivo*. To study the retention of thymol and its two glucosides in the lumen of the GIT of pigs, animals were fed with these compounds for 1 day in discrete meals every 2 h. All piglets consumed the total amount of feed offered on the day of sampling. The additive concentrations in the feed (expressed on fresh matter basis) were analytically checked and found to differ slightly from the intended dose (3333 μmol

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ACS Omega XXXX, XXX, XXX−XXX

Table 2. Accuracy (%), Precision (RSD%), and Recovery (%) of Thymol and Thymol α-D-Glucopyranoside in Different Matrices

	spiked concentration (mg kg^{-1})					
	thymol			thymol α-D-glucopyranoside		
matrix	50	100	500	50	100	500
feed						
accuracy (n = 9)	91.5	90.6	84.7	87.6	98.0	95.2
intraday precision (n = 3)	4.1	2.8	7.5	4.6	3.2	3.9
interday precision (n = 3)	12.7	4.0	13.1	9.8	8.2	6.9
recovery (n = 3)	101.3	118.0	113.1	105.3	122.3	115.2
gastric digesta						
accuracy (n = 9)	103.2	96.0	92.2	108.2	106.3	98.6
intraday precision (n = 3)	4.9	5.7	5.2	6.5	5.3	2.7
interday precision (n = 3)	6.1	8.4	5.4	9.1	7.5	3.4
recovery (n = 3)	103.2	114.6	95.3	106.3	116.4	98.2
small intestinal digesta						
accuracy (n = 9)	102.3	94.7	95.5	99.1	98.2	96.1
intraday precision (n = 3)	11.9	6.7	8.4	1.4	1.4	2.2
interday precision (n = 3)	13.4	7.5	10.5	3.4	2.3	2.6
recovery (n = 3)	110.6	103.9	105.7	96.5	96.1	100.1
cecal digesta						
accuracy (n = 9)	109.3	105.9	107.6	99.9	100.4	99.7
intraday precision (n = 3)	5.1	5.9	11.5	6.1	4.3	0.5
interday precision (n = 3)	10.4	6.3	15.4	6.1	4.9	0.9
recovery (n = 3)	104.4	112.0	98.6	101.0	96.7	94.8

kg^{-1}). The detected values were 3603 μmol thymol kg^{-1} for CON, 2790 μmol TαG kg^{-1} plus 149 μmol free thymol kg^{-1} for αGLUC, and 3369 μmol TβG kg^{-1} for βGLUC. This corresponds to a total thymol dose of 108, 88, and 101 μmol kg BW^{-1} for the respective treatments. Further calculations were based on the analytically verified concentrations in the feed and expressed on DM basis to be able to compare across compartments.

The detected concentrations of thymol, TαG, and TβG in gastric contents of piglets 1 to 2 h post prandial condition are presented in Table 3. Piglets orally supplemented with pure thymol (CON) retained concentration levels in their stomach that were, on average, 75.3% of the concentration present in the feed (p < 0.001; Figure 2). When piglets were fed with the monoglucosides TαG or TβG, the total thymol concentrations (sum of free and glucoconjugated thymol) in stomach contents were respectively 71.4% (p < 0.001) and 48.1% (p = 0.001) of the original feed levels. Additionally, the total thymol concentration at the level of the stomach was significantly lower in βGLUC (1820 μmol kg^{-1} DM) as compared to CON piglets (3048 μmol kg^{-1} DM; p = 0.002), with αGLUC piglets showing intermediate levels (2357 μmol kg^{-1} DM). The proportion of the intact glucosides in the total thymol concentrations were 66 and 63% for the αGLUC or βGLUC treatment, respectively, while the remaining part was present as free thymol. This finding implicates that the glucosides are partially hydrolyzed in the stomach before absorption. Additionally, there was a trend (p = 0.069) for lower intact glucoside concentrations in the stomach of βGLUC pigs (1149 μmol kg^{-1} DM) compared to αGLUC pigs (1556 μmol kg^{-1} DM).

Only trace amounts (<LOQ) of free thymol were found in small intestinal and cecum contents of pigs of either treatment. Furthermore, no detectable levels of the glucosides were found further in the GIT of the αGLUC or βGLUC piglets.

Table 3. Analyte Concentrations in Feed and the Stomach Contents of Piglets of Different Treatments (μmol kg^{-1} Dry Matter)

analyte	feed	stomach[a]	p-value[b]
CON[c]			
free thymol	4049	3048 ± 132	<0.001
TαG[c]	ND[d]	ND	
TβG[c]	ND	ND	
total thymol	4049	3048 ± 132	<0.001
αGLUC[c]			
free thymol	167	801 ± 109	0.001
TαG[c]	3135	1556 ± 133	<0.001
TβG[c]	ND	ND	
total thymol	3302	2357 ± 136	<0.001
βGLUC[c]			
free thymol	ND	672 ± 156	
TαG[c]	ND	ND	
TβG[c]	3786	1149 ± 150	<0.001
total thymol	3786	1820 ± 283	0.001

[a]Expressed as mean ± SE. [b]Generated via a one-sample t-test (n = 6) with feed concentration as the test value. [c]Abbreviations: CON, control treatment with an intended dose of 3333 μmol of thymol per kg of feed; αGLUC, treatment with an intended dose of 3333 μmol of thymol α-D-glucopyranoside per kg of feed; βGLUC, treatment with an intended dose of 3333 μmol of thymol β-D-glucopyranoside per kg of feed; TαG, thymol α-D-glucopyranoside; TβG, thymol β-D-glucopyranoside. [d]ND, not detected; value below limit of detection.

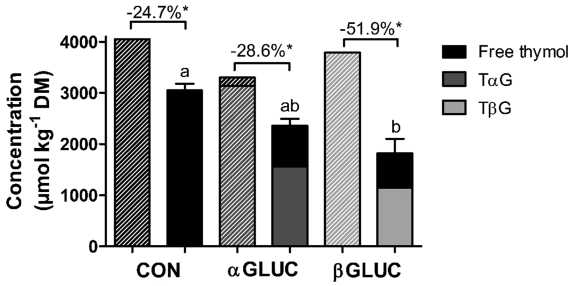

Figure 2. Concentrations of analytes in feed (striped bars) and stomach contents (full bars) of piglets fed with thymol (CON), thymol α-D-glucopyranoside (αGLUC), or thymol β-D-glycopyranoside (βGLUC). Error bars indicate the SEM (n = 6) in stomach contents. Percentages reflect differences in total thymol concentrations (sum of free and glucoconjugated thymol). Asterisks (*) indicate significant differences, which were tested via a one-sample t-test with the corresponding feed concentration as the test value. Different letters (a, b) indicate a significant difference in total thymol concentration of stomach contents between treatments tested with a one-way ANOVA procedure (p = 0.002).

DISCUSSION

To evaluate the retention of thymol and TαG from the GIT of piglets, it was essential to develop a reliable method to extract these compounds from and quantify them in feed and digesta of different gastrointestinal sites. With one exception,[22] most

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ACS Omega XXXX, XXX, XXX−XXX

published methods for the analysis of thymol conjugates[7,20,23] involve a first step of enzymatic hydrolysis. The amount of conjugates is then indirectly calculated from the difference in thymol concentration before and after hydrolysis. The accuracy of indirect methods is highly dependent on the selectivity and progression of the enzymatic reactions.[24] The method proposed in this study avoids indirect hydrolysis and proved to fulfill all set requirements for validation. The sensitivity was judged to be sufficiently high for quantification of the analytes in digesta of this particular *in vivo* trial. If no absorption would occur, then the dietary levels are diluted 4 times in gastric contents (mean DM content of 25%) and 10 times in small intestinal contents (mean DM content of 10%). Taking into account the administered doses of thymol (500 mg kg^{-1}) and TαG (1040 mg kg^{-1}), the expected maximal concentrations amount approximately 125 and 260 mg kg^{-1} in stomach and 50 and 104 mg kg^{-1} in small intestine for thymol and TαG, respectively. These values are all above the LOQ. However, it should be mentioned that, when applying lower doses, the sensitivity of this method might be insufficient. In this case, the remaining concentrations in the GIT are also below the range of minimum inhibitory concentrations against several pathogens[1] and thus functionally not relevant for our purpose.

The major drawback for the use of thymol as an antimicrobial compound in live animals is its fast clearance from the upper GIT. Therefore, the luminal concentrations in the distal small intestine remain below the bactericidal thresholds.[7,8] The lower thymol concentrations retrieved from the stomach contents of thymol-fed piglets as compared to the feed and the absence of quantifiable amounts in digesta from the small intestine in this study confirm these findings. Glucoconjugation of the monoterpene has been tested here as a way to deliver higher concentration to the lower gut region. Glucosides are more hydrophilic and have a larger molecular size, which makes them more resistant to penetration of epithelial membranes.[25] Moreover, the pungent taste of the aglycon is masked and its volatility reduced by this glucoconjugation. However, it was shown by Epps et al.[26] and Levent et al.[27] that TβG has no antimicrobial activity and that its ability to reduce bacterial populations depends on the presence of glucoside-hydrolyzing enzymes to release the active aglycon. The abundance of glucosidase enzymes in the proximal GIT is thought to be rather low. On the contrary, the high level of glucosidase activity in the distal GIT should release the active aglycon at the target sites.[15,28] In the current study, however, the total thymol concentration remaining in the stomach of piglets fed with TαG or TβG was significantly lower than that in the feed, indicating that some absorption already took place under the assumption that thymol as such is not broken down by microbiota in the foregut.[7] Moreover, feeding TβG resulted in total thymol concentrations in the stomach that were 40% lower than in the thymol treatment. Supplementing TαG gave intermediate total stomach concentrations. Hence, feeding glucoconjugates of thymol did not result in higher luminal thymol concentrations in the stomach as compared to supplementation of free thymol. On the contrary, the β-glucoside form seemed to disappear faster from the chyme than its aglycon. This is in agreement with the study of Cermak et al.,[29] who administered an equimolar dose of quercetin or its β-glucoside isoquercitrin to pigs and registered a 50% increase in bioavailability from the glucoside as compared to the aglycon. Moreover, for both quercetin and isoquercitrin, the main metabolite appeared in the blood circulation within 1 h after administration, but the peak of the latter was higher. This indicated that isoquercitrin was absorbed faster, but still from the same site, namely, the upper GIT.[29] Actually, intact absorption at the level of the stomach has been suggested for some glucoconjugated anthocyanidins without prior hydrolysis,[30,31] although this was not the case for the flavonoid glucosides of quercetin[32] and daidzein.[33] Therefore, it remains to be elucidated whether absorption of glucoconjugated thymol through the gastric wall contributes to the reduced total thymol concentration as compared to aglycon supplementation. In any case, the observed thymol glucoconjugate clearance from the upper GI tract does not favor the antimicrobial action in the hindgut, yet it might promote the systemic antioxidant[34] or anti-inflammatory activity.[35]

In the stomach contents of αGLUC and βGLUC piglets, more than 30% of the total thymol was present in the free form, suggesting that hydrolysis of both TαG and TβG took place in the stomach. Additionally, the intact TαG concentration in stomach tended to be higher as compared to TβG ($p < 0.10$), despite the slightly lower TαG concentration in feed. This implies that the α-bound conjugates might be more resistant to hydrolysis and absorption than the β-bound ones. Early glucoside hydrolysis was also suggested by Epps et al.,[21] who found reduced counts of *Campylobacter* in the crop of broilers fed with TβG. As mentioned before, intact TβG has no antimicrobial activity, so hydrolysis and release of the biologically active aglycon must have occurred in the crop. By contrast, Crespy et al.[32] measured virtually unaltered concentrations of isoquercitrin in the rat stomach 30 min after gastric administration, while 38% of the administered aglycon, quercetin, was absorbed. The authors concluded that the stomach does not play a crucial role in absorption nor hydrolysis of glucosides. Important differences of this rat study with our study might be the absence of feed in the stomach and the lack of contact between the compounds and saliva as the compounds were administered intragastrically. Indeed, Walle et al.[36] demonstrated the hydrolysis of quercetin, phloretin, and genistein glucosides by human saliva. It is rather implausible that these monoglucosides were hydrolyzed by salivary α-amylases as these enzymes are endosaccharidases that have no effect on terminal glucose molecules,[37] although the required glucosidase activity most likely originated from bacteria and shed epithelial cells present in the oral cavity.[36] Otherwise, gastric bacteria might also contribute to glucoside hydrolysis. Unlike humans, piglets harbor relatively large numbers of bacteria in their stomach, among which are *Lactobacilli* and *Enterococci,* who are known to exhibit glucosidase activities.[38−40] Hydrolysis due to the low pH in the stomach does not probably add to the appearance of the aglycon as the temperature requirements for significant acid hydrolysis are much higher than physiological temperatures.[41,42] Deconjugation of the glucosides in the feed, prior to ingestion, might also be suggested as the presence of glucosidase activity has been shown in many plants commonly used as feed ingredients such as maize, soybean, and wheat.[43] The occurrence of free thymol in the current study is not likely attributable to in-feed hydrolysis because the compounds were mixed in the feed only 1 day prior to feeding. The minor fraction of free thymol that was present in the αGLUC feed originates from an impurity of the TαG product. Hence, the occurrence of free aglycon levels in the stomach contents is thought to originate mainly from bacterial activity in both

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ACS Omega XXXX, XXX, XXX−XXX

saliva and stomach, although further research is warranted to confirm this hypothesis and to elucidate why the β-glucoside tends to be more easily hydrolyzed than its α-isomer.

As expected, free thymol levels in the small intestine and cecum were very low, even below the quantification limit, irrespective of the treatment. This is largely in accordance with the findings of Michiels et al.,[7] although they still retrieved a minor fraction of the ingested essential oils in SI1. This difference might be explained by the way of administration: a single dose versus spread in meals in our study and by the application of another extraction and analysis method. Surprising was the inability to detect any glucosides in digesta of αGLUC and βGLUC piglets as of SI1 in our study. Similar results were obtained by feeding black raspberry powder, containing cyanidin-3-glucoside among other glycosides to weaner pigs. Four hours after the meal, the monoglucoside almost completely disappeared from small intestinal contents, and only 2% of the total intake was recovered in cecum and colon.[44] We assume that the amount of monoglucosides remaining in the chyme must be absorbed almost instantaneously in the duodenum upon gastric emptying, as is the case for thymol. Although, to date, no studies are available that deal with the absorption route of thymol glucosides, much research has been done on quercetin glucosides. With regard to the absorption of monoglucosides across the intestinal membrane, two hypotheses exist. First, glucosides might be hydrolyzed by small intestinal glucosidases, such as the membrane-bound lactase phlorizin hydrolase, before trans-epithelial absorption of the aglycon by passive diffusion occurs.[45,46] Second, there might be carrier-mediated uptake of the monoglucoside as such by the sodium-dependent glucose transporter-1.[47] Moreover, the bioavailability and the rate of small intestinal absorption of quercetin were demonstrated to be highly dependent on the glycoside moiety to which it is conjugated. Indeed, glucose conjugates were rapidly absorbed, while other glycosides, such as galactose, rhamnose, and glucorhamnose, were more resistant to intestinal hydrolysis and absorption.[29,48] Hence, it could be of interest to synthesize and investigate the potential of thymol conjugates with glycons other than glucose or with more than one sugar unit to obtain delayed absorption of the aglycon.

In conclusion, we observed that glucoconjugation did not protect thymol from early absorption in the proximal GIT of piglets. Supplementation of TβG even seemed to increase the absorption rate in the stomach as compared to the aglycon, as appeared from the 40% lower total thymol concentrations in the chyme. Importantly, it was shown that both thymol glucosides are subject to partial hydrolysis in the stomach, yet TαG seemed more resistant than TβG. Although the contribution of bacteria from the oral cavity and stomach is plausible, the mechanisms of glucoside hydrolysis in the stomach need further investigation.

MATERIALS AND METHODS

Chemicals. Thymol (99.5% purity) was obtained from Sigma-Aldrich (Bornem, Belgium). Thymol α-D-glucopyranoside (97% purity, 0.8% free thymol) was enzymatically synthesized according to the procedure described by De Winter et al.[18] from a reaction mixture containing thymol (5 g L^{-1}) and sucrose (1 mol L^{-1}) and facilitated by the R134A mutant of *Thermoanaerobacterium thermosaccharolyticum* sucrose phosphorylase (4 U mL^{-1}). Thymol β-D-glucopyranoside (99.4% purity) was purchased from Glentham Life Sciences Ltd. (Corsham, U.K.). The internal standards 2-isopropylphenol (≥98% purity) and 4-nitrophenyl α-D-glucopyranoside (99.8% purity) were purchased from Fluka (Bornem, Belgium) and Carbosynth Ltd. (Berkshire, U.K.), respectively. All organic solvents were of HPLC grade and water was of Milli-Q quality (Merck Millipore, Darmstadt, Germany).

Standard Solutions and Calibration Curves. Thymol and TαG were accurately weighed in the same tube and subsequently dissolved in ethanol to obtain a stock solution containing 24 g L^{-1} of both analytes. The standard solutions were made by further diluting the stock solution with the appropriate amounts of ethanol. A separate calibration curve was constructed for following matrices: feed and digesta from stomach, small intestine, and cecum. The calibration curves were obtained by adding the standard solutions to blank samples, resulting in final thymol and TαG concentrations of 5, 25, 50, 100, 250, 500, 1000, and 1200 mg kg^{-1}. The blank samples from gastric, small intestinal, and cecal digesta for matrix-matched calibration curves and the assessment of validation parameters were pooled samples obtained from pigs fed with a diet free from any thymol compounds. Two internal standard solutions were prepared: 2-isopropylphenol (iPP; 25 g L^{-1}) for thymol[7] and 4-nitrophenyl α-D-glucopyranoside (pNPG; 0.5 g L^{-1}) for TαG.

Sample Preparation. All samples were extracted and analyzed in triplicate, unless stated otherwise. Samples were acidified to pH < 2 with 2% 6 mol L^{-1} H_2SO_4 solution to prevent fermentation before adding the test compounds (validation procedure) or at sample collection (*in vivo* trial). The liquid−liquid extraction procedure was adapted from Gallo et al.[49] One gram of sample was weighed in a 15 mL glass vial with a screw cap with septum. Next, each sample was spiked with 50 μL of iPP and pNPG internal standards. Thereafter, 2 mL of the extraction solvent consisting of ethyl acetate, 1-butanol, and 1-propanol (60:30:10, v/v/v) was added. The samples were first vigorously mixed on a vortex apparatus for 30 s and then shaken horizontally on an orbital shaker (300 rpm) for 1 h, followed by centrifugation (2000*g*, 10 min) and transfer of the organic top layer. The extraction procedure was repeated a second time following 30 min shaking. Subsequently, the organic phase was evaporated to dryness under nitrogen gas at 40 °C. The dry residue was reconstituted in 1 mL of a mixture of water and acetonitrile (65:35, v/v) and filtered with a cellulose syringe filter (0.2 μm). Aliquots of 20 μL were injected in the HPLC system upon analysis.

Chromatographic Condition. Liquid chromatography was performed on an Agilent Technologies 1200 series LC system. Chromatographic separation was acquired on a reversed-phase C18 column (Supelcosil LC-18, 5 μm, 25 cm × 4.6 mm i.d.) maintained at 35 °C and protected by a Supelguard cartridge (Supelcosil LC-18, 5 μm, 2 cm × 4 mm i.d.). Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), both with 1 mL L^{-1} formic acid. The elution gradient was set as follows [time in minutes (% B)]: 0 (5), 5 (5), 25 (60), and 30 (100), with a flow rate of 2 mL min^{-1}. Thymol, TαG, and TβG were detected at the wavelength of 280 nm.

Method Validation. The method was validated according to the International Conference of Harmonization (ICH) Guideline for the Validation of Analytical Procedures. For each biological matrix (feed, gastric, small intestinal, and cecal contents), the following validation parameters were evaluated:

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ACS Omega XXXX, XXX, XXX−XXX

selectivity, linearity, precision and accuracy, recovery, limit of detection (LOD), and limit of quantification (LOQ). Because of budget constraints, the method was only validated for thymol and TαG, not for TβG.

Selectivity. Selectivity was evaluated by comparing chromatograms of nine blank samples of each matrix with corresponding spiked samples and testing for peak interferences.

Linearity. For the linearity study, blank samples were spiked simultaneously with thymol and TαG, as described in the previous section (standard solution and calibration curves). The calibration curves consisted of blank samples and eight concentration levels ranging from 5 to 1200 mg kg^{-1} of both analytes. This range was chosen based on the expected concentrations in the samples of the current *in vivo* experiment. The dependent variable in the linear regression analysis was calculated as the ratio of peak area of the analyte/peak area of its respective internal standard. Linearity was evaluated using linear regression analysis calculated with the least-squares method.

Accuracy, Precision, and Recovery. Accuracy, within- and between-day precision, and recovery were determined by analyzing spiked samples at three different concentrations: low (50 mg kg^{-1}), medium (100 mg kg^{-1}), and high (500 mg kg^{-1}). The analysis was performed in triplicate and on three consecutive days by the same analyst (interday precision) for every matrix. Accuracy was calculated as the ratio between the mean measured concentration and the nominal concentration multiplied by 100, while precision was expressed as the relative standard deviation (RSD) of the measured concentrations. Concentrations were calculated from the obtained peak areas using the previously mentioned calibration curves. Recovery was determined by comparing the response of the samples spiked before extraction with the response from blank samples that were spiked after extraction and drying. The response is defined as the ratio between the peak areas of the analyte and the respective internal standard.

Sensitivity. Sensitivity is determined by the LOD and LOQ. For this purpose, a separate eight-point calibration curve was constructed in the lower concentration regions (1−25 mg kg^{-1}) for each matrix. LOD and LOQ were calculated as the ratio of the RSD of the regression line and the slope of the calibration curve multiplied by 3.3 and 10, respectively.

***In Vivo* Absorption of Thymol and Its Glucosides.**

Animal Housing and Treatments. The study was conducted in accordance with the ethical standards and recommendations for accommodation and care of laboratory animals covered by the European Directive 2010/63/EU on the protection of animals used for scientific purposes and the Belgian royal decree KB29.05.13 on the use of animals for experimental studies.

Eighteen female piglets (Topigs × Piétrain) with a mean body weight (BW) of 24.0 ± 0.6 kg were selected from a herd and housed per two in a pen with full slated floors. The stable had a conventional ventilation scheme, ambient temperature at 24 °C, and a 18 light/6 dark schedule. The animals were allocated to treatments (n = 6) with stratification for BW. Subsequently, the pens were assigned to the treatments according to a randomized block design. Piglets were adapted to meal feeding for 7 days during which they received an appropriate basal diet for growing piglets (Table 4), provided in eight equal meals per day between 6 a.m. and 8 p.m. This approach was used to mimic steady-state conditions in the GIT. The total daily feed intake was restricted to 40 g kg^{-1} BW (equivalent to approximately 90% of the ad libitum feed intake). Animals were allowed to eat for 30 min per meal, after which the residual feed was collected and weighed. From day 6 onward, all animals consumed the total amount of feed provided. Water was available ad libitum during the whole experiment. On the day of sampling (day 8), animals received six meals of their respective experimental diets with a 2 h interval and were finally euthanized between 1 and 2 h after their last meal. Experimental diets consisted of the basal diet supplemented with 3333 μmol kg^{-1} thymol (equivalent to 500 mg kg^{-1}; CON treatment), an equimolar amount of thymol-α-D-glucopyranoside (1040 mg kg^{-1}; αGLUC treatment), or thymol-β-D-glucopyranoside (1040 mg kg^{-1}; βGLUC treatment).

Table 4. Ingredient and Analyzed Nutrient Composition of the Basal Diet (g kg^{-1} as Feed)

ingredient composition	
wheat	355.00
barley	300.00
soybean meal	136.22
corn	80.00
toasted soybeans	40.00
sugarbeet pulp	20.00
animal fat	19.94
premix minerals and vitamins[a]	5.00
soybean oil	10.00
L-lysine	9.25
dicalciumphosphate	8.02
limestone	6.96
L-threonine	2.28
salt	2.00
sodium bicarbonate	1.95
DL-methionine	1.62
L-valine	1.17
L-tryptophan	0.61
analyzed nutrient composition	
dry matter	892.9
crude ash	47.3
crude protein	182.0
ether extract	54.5
crude fiber	36.0

[a]Providing per kg of diet: vitamin A (retinyl acetate), 15,000 IU; vitamin D3 (cholecalciferol), 2000 IU; vitamin E (all-*rac*-α-tocopherylacetate), 50.0 mg; vitamin K3 (menadion), 4.0 mg; vitamin B1 (thiamine mononitrate), 3.1 mg; vitamin B2 (riboflavin), 8.0 mg; vitamin B3 (calcium-D-pantothenate), 20 mg; vitamin B6 (pyridoxine hydrochloride), 6.0 mg; vitamin B12 (cyanocobalamin), 50.0 μg; vitamin PP (niacinamide), 40.0 mg; folic acid, 2.0 mg; biotin, 0.3 mg; betaine anhydrate, 285 mg; endo-1,4-β-glucanase E3.2.1.4, 250 TGU; endo-1,4-β-xylanase E3.2.1.8, 560 TXU; 6-phytase, 500 OTU; Fe (iron(II)sulfate monohydrate), 24.0 mg; Cu (copper(II)sulfate pentahydrate), 155.0 mg; Zn (Zn MHA), 100.0 mg; Mn (manganese-(II)oxide), 48.0 mg; I (calcium iodate anhydrate), 1.9 mg; Se (sodium selenite), 200 μg; Se (selenomethionine produced by *Saccharomyces cerevisiae* NCYC-R397), 100 μg; E306 extract of vegetable oils rich in tocopherols, 228 mg; clinoptioliet, 1.64 g; aromatic compounds, 72 mg.

Sample Collection and Analysis. Animals were first brought to electronarcosis followed by exsanguination. The entire GIT was removed, exposed, and partitioned in five digesta sampling sites: stomach, three parts of the small

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intestine (SI1, SI2, and SI3 corresponding to segments of 0−25%, 25−75%, and 75−100% of the total length, respectively), and cecum. The contents of each compartment were quantitatively collected. An aliquot of the fresh digesta was acidified to pH < 2 with 2% 6 mol L^{-1} H_2SO_4 and stored at −20 °C. These samples were further processed as described earlier for determination of the concentration of thymol and its glucosides. The remaining intestinal contents were frozen and freeze-dried to determine dry matter (DM) contents. Feed samples were collected to verify the concentrations of the analytes.

Statistical Analysis. Data analysis was performed with SPSS Statistics 24.0 program (SPSS Inc., Chicago, USA). Assumptions of normality and equality of variances were checked using the Shapiro−Wilkinson and Levene tests, respectively. Results were analyzed using *t*-tests and ANOVA procedures on the 5% significance level.

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Notes

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ABBREVIATIONS

αGLUC, treatment with an intended dose of 3333 μmol of thymol α-D-glucopyranoside per kg of feed; βGLUC, treatment with an intended dose of 3333 μmol of thymol β-D-glucopyranoside per kg of feed; BW, body weight; CON, control treatment with an intended dose of 3333 μmol of thymol per kg of feed; DM, dry matter; GIT, gastrointestinal tract; iPP, 2-isopropylphenol; LOD, limit of detection; LOQ, limit of quantification; pNPG, 4-nitrophenyl α-D-glucopyranoside; RSD, relative standard deviation; SI1, small intestinal segment from 0−25% of the total length; SI2, small intestinal segment from 25−75% of the total length; SI3, small intestinal segment from 75−100% of the total length; TαG, thymol α-D-glucopyranoside; TβG, thymol β-D-glucopyranoside

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